Description

SODIUM/PROTON ANTIPORTER GENE

5   <u>Cross-Reference to a Related Application</u>

This application is a continuation-in-part of PCT/JP99/07224 filed December 22, 1999; which claims priority to Japanese Application No. 10/365604, filed December 22, 1998.

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<u>Technical Field</u>

The present invention relates to a novel Na^+/H^+ antiporter derived from plants and the DNA encoding the antiporter, as well as methods for producing and using the same.

15

<u>Background Art</u>

Salt tolerance of plants is important to both agriculture and environmental protection. Today, one third of the land on earth is said to be dry land. Further, it is anticipated that
20   the proportion of dry land will increase in the future, due to the progressive desertification of both cultivated land and green land. Considering the prediction that the world population in the year 2050 will be 1.5 times that of today and the serious problems of provisions arising as a result, development of
25   cultivars that grow on land ill-fitted for cultivation, especially on dry land, as well as cultivation techniques for the same is a matter of great urgency. The problem with agriculture on dry land is salt accumulation. In a dry climate, evapotranspiration outstrips precipitation and continued
30   irrigation on land where much is desired for drainage leads to plenty of salt accumulation, due to the deposition of salt on the surface by acceleration of rise in subterranean water level that bear salinity. Examples where cultivation becomes

impossible as a result are known from the ancient past, represented by the end of Tigris-Euphrates civilization. The problem still arises today.   Thus, innovation of agriculture, on dry land and on land where salt is accumulated, to enhance

5   the salt tolerance of plants is of great importance (Toshiaki Tanno (1983) Kagaku to Seibutsu 21:439-445 "Salt tolerance of crops and mechanism of the same"; Yasutaka Uchiyama (1988) Kagaku to Seibutsu 26:650-659 "Agricultural use of salinenvironment").

There are two kinds of stress related to salt stress against

10   plants, namely stress by osmotic pressure and stress by ionicity. An osmotic pressure stress is a stress whose action is the same as the stress by dehydration.   It results from high osmotic pressure, due to high salinity environment around the plant, which leads to a setback of water absorption of the plant and

15   at the same time deprivation of water from the plant body.   It is known that a mechanism exists in the plant to avoid the osmotic pressure stress.   The core substances associated with this function are ions (such as K^+, Na^+, Cl^-, organic acid, etc.) as well as substances called compatible solutes.   The term

20   "compatible solute" refers to substances such as sugar, proline (a kind of amino acid), and glycine betaine (a quaternary ammonium compound), and so on, which do not disturb the metabolic pathway or inhibit enzymatic action, even when accumulated at a high concentration in the cell.   Plant cells accumulate these

25   substances which, in turn, preserve the osmotic pressure balance to the external world (Manabu Ishitani, Keita Arakawa, and Tetsuko Takabe (1990) Chemical Regulation of Plants 25:149-162, "Molecular mechanism of salt tolerance in plants").

Almost no development has been made regarding the mechanism

30   of plants to avoid ionic stress.   Absorption of excess Na^+ by the plant cell leads to inhibition of intracellular enzyme reaction and finally to metabolic trouble (Toru Matoh (1997) Chemical Regulation of Plants 32:198-206, "Salt tolerance

mechanism of the plant"). Therefore, it is necessary to eliminate the intracellulary accumulated Na^+ from the cell or isolate it into intracellular organs, such as vacuoles. The Na^+/H^+ antiporter (sodium/proton antiporter) is assumed to play

5   the central role in this process. The Na^+/H^+ antiporters of plant cells are thought to exist on both the cell membrane and the vacuolar membrane. They utilize the pH gradient formed between the biomembranes by the H^+ pump (H^+-ATPase and H^+-PPase), an element that transports H^+ as the energy to transport Na^+ existing

10   in the cytoplasm out of the cell or into the vacuole. Moreover, it is presumed that plants contacted with salt of high density, have to retain intercellular K^+/Na^+ ratio high enough, maintaining the osmotic pressure balance between the cell exterior and interior by accumulating Na^+ in the vacuole through

15   the Na^+/H^+ antiporter.

The Na^+/H^+ antiporters found existing on plasma membrane are well examined in animals, yeasts, bacteria and so on. On the plasma membrane of an animal cell, H^+ is carried by the Na^+/H^+ antiporter, to maintain the balance of H^+ in the cell, utilizing

20   the Na^+ concentration gradient between the membranes formed by Na^+/K^+-ATPase. Therefore, the antiporter is presumed to be deeply related with intracellular pH modulation, control of the cell volume, as well as Na^+ transport through the plasma membrane (Orlowski, J. and Grinstein, S. (1997) J.Biol.Chem.

25   272:22373-22376; Aronson, P.S. (1985) Ann.Rev.Physiol. 47:545-560). Na^+/H^+ antiporters exist in various cells of animals and six isoforms (NHE 1 to 6) have been reported (Orlowski, J. and Grinstein, S. (1997) J.Biol.Chem 272:22373-22376).

The first gene cloned for yeast was the gene (sod2) from

30   fission yeast (*Schizosaccharomyces pombe*), which was cloned as a gene related to Na^+ transport and salt tolerance (Jia, Z.P., McCullough, N., Martel, R., Hemmingsen, S. and Young, P.G. (1992) EMBO J. 11:1631-1640). Also, a gene with high identity to this

gene has been found from a budding yeast (*Saccharomyces cerevisiae*), as well as *Zygosaccharomyces rouxii* (named NHA1 and ZSOD2, respectively) (Prior, C. et al. (1996) <u>FEBS Letter</u> <u>387</u>:89-93; Watanabe, Y. *et al.* (1995) <u>Yeast</u> <u>11</u>:829-838). Two

5   different Na^+/H^+ antiporter genes (nhaA, nhaB) have been isolated from *E.coli* (Karpel, R. *et al.* (1988) <u>J.Biol.Chem.</u> <u>263</u>:10408-10410; Pinner, E. *et al.* (1994) <u>J.Biol.Chem.</u> <u>269</u>:26274-26279), each closely related to Na^+ transport and salt tolerance. ·With respect to plants, activities in algae and such

10   have been examined (Katz, A. *et al.* (1989) <u>Biochim.Biophys.Acta</u> <u>983</u>:9-14).

On the other hand, there are only reports on activity in plants for antiporters restricted on vacuolar membranes. To date, Na^+/H^+ antiporters on the vacuoles have been investigated

15   in connection with salt tolerance in halophytes growing in an environment with high salinity (Matoh, T. *et al.* (1989) <u>Plant</u> <u>Physiol.</u> <u>89</u>:180-183; Hassidim, M. *et al.* (1990) Plant Physiol. <u>94</u>:1795-1801; Barkla, B.J. *et al.* (1995) <u>Plant Physiol.</u> <u>109</u>:549-556), as well as in glycophytes with high salt tolerance,

20   like barley and sugar beet (Hassidim, M. *et al.* (1990) <u>94</u>:1795-1801; Blumwald, E. *et al.* (1987) <u>Plant Physiol.</u> <u>85</u>:30-33; Garbarino, J. and DuPont, F.M. (1988) <u>Plant Physiol.</u> <u>86</u>:231-236; Garbarino, J. and DuPont, F.M. (1989) <u>Plant Physiol.</u> <u>89</u>:1-4; Staal, M. *et al.* (1991) <u>Physiol.Plant.</u> <u>82</u>:179-184). The above

25   findings indicate that Na^+/H^+ antiporters are closely related to salt tolerance of plants. There are several reports on characteristics of Na^+/H^+ antiporters on the vacuolar membrane. The Km of the antiporter activity for Na^+ is about 10mM similar to that on cytomembrane of mammals (Blumwald, E. *et al.* (1987)

30   <u>Plant Physiol.</u> <u>85</u>:30-33; Garbarino, J. and DuPont, F.M. (1988) <u>Plant Physiol.</u> <u>86</u>:231-236; Orlowski, J. (1993) <u>J.Biol.Chem.</u> <u>268</u>:16369-16377). Moreover, it is known that amiloride and amiloride derivatives, which are specific inhibitors of Na^+

transporters, inhibit the Na^+/H^+ antiporters on the plant
vacuolar membrane and that on the mammalian plasma membrane in
a competitive manner (Blumwald, E. *et al.* (1987) <u>Plant Physiol.</u>
<u>85</u>:30-33; Orlowski, J. (1993) <u>J.Biol.Chem.</u> <u>268</u>:16369-16377; Tse,
C.M. *et al.* (1993) <u>J.Biol.Chem.</u> <u>268</u>:11917-11924; Fukuda, A. *et*
al. (1998) <u>Plant Cell Physiol.</u> <u>39</u>:196-201). These findings
suggest the characteristic similarities between Na^+/H^+
antiporter on the vacuolar membrane of plants and that on
mammalian plasma membrane. There are various reports on Na^+/H^+
antiporter activity of plants as mentioned above, however, in
spite of the various trials done, analysis of the substantial
part, namely genes as well as proteins thereof, were still left
behind (Katz, A. *et al.* (1989) <u>Biochim.Biophys.Acta</u> <u>983</u>:9-14;
Barkla, B. and Blumwald, E. (1991) <u>Proc.Natl.Acad.Sci.USA</u>
<u>88</u>:11177-11181; Katz, A., Kleyman, T.R., and Pick, U. (1994)
<u>Biochemistry</u> <u>33</u>:2389-2393).

Recently, a gene expected to encode a protein that shares
amino acid sequence homology with known Na^+/H^+ antiporter has
been cloned from *Arabidopsis*; however, the function of this gene
remains to be resolved (M.P. Apse *et al.* (1998) Final Programme
and Book of Abstracts "11th International Workshop on Plant
Membrane Biology", Springer; C.P. Darley *et al.* (1998) Final
Programme and Book of Abstracts "11th International Workshop
on Plant Membrane Biology", Springer).

Examples of Na^+/H^+ antiporter genes isolated from plants
are only those isolated from *Arabidopsis*, a dicotyledon,
described above. No isolation of genes from monocotyledoneae,
including species such as rice and maize, which are industrially
useful crops, have been reported until now.

Disclosure of the Invention

Of all the important crops, rice is a crop with low salt
tolerance. Its growth is inhibited to the halves with 150mM NaCl

as compared to barley, which is a highly salt tolerant crop, and shows inhibition of the same level with 250 mM NaCl. Garbarino *et al.* reported the suppression of Na^+ flow to the shoot by accumulating Na^+ in the vacuole of the root might increase

5   salt tolerance of barleys (Garbarino, J. and DuPont, F.M. (1988) Plant Physiol. 86:231-236). From verifying this fact, it has been known that the Na^+/H^+ antiporter activity of the barley root vacuolar membrane increases through treatment with salt. It has also been known that barley has far and away a higher activity

10   than rice (Garbarino, J. and DuPont, F.M. (1988) Plant Physiol. 86:231-236; Fukuda, A. Yazaki, Y., Ishikawa, T., Koike, S., and Tanaka, Y. (1998) Plant Cell Physiol. 39:196-201).

On the contrary, the activity does not rise in rice even if it is treated with salt (Fukuda, A. Yazaki, Y., Ishikawa,

15   T., Koike, S., and Tanaka, Y. (1998) Plant Cell Physiol. 39:196-201). Further, Na^+ transport from root to the shoot of rice is known to be higher than that of the phragmites, which belong to the *Gramineae* family, like rice, and shows higher salt tolerance (Matsushita, N. and Matoh, T. (1991) Physiol.Plant.

20   83:170-176). Therefore, it is possible that the strength of Na^+/H^+ antiporter activity of the root vacuolar membrane is deeply associated with rice salt tolerance. These reports indicate that it might be possible to increase salt tolerance of rice by rising Na^+/H^+ antiporter activity in the rice root. On this

25   account, there was a desire to isolate genes that might increase Na^+/H^+ antiporter activity of rice.

This situation led to the present invention, an object of which is to provide an Na^+/H^+ antiporter derived from monocotyledoneae, preferably rice, and gene(s) encoding the same,

30   as well as a method for producing and using the same. The present invention provides use of the gene for production of salt tolerant plants as a favorable use of the present DNA.

The present inventors identified a cDNA clone from rice

anthotaxy that shares homology with the Na⁺/H⁺ antiporter (*NHX1*) gene from the budding yeast by analyzing a base sequence from the GeneBank higher plants database. Using this sequence as a probe, the present inventors succeeded in newly cloning the full-length gene designated "*OsNHX1*", which is expected to encode the Na⁺/H⁺ antiporter of rice.

The isolated *OsNHX1* cDNA is approximately 2.3kb and is presumed to encode a protein of 535 amino acids (Figure 1). From an amino acid hydrophobicity analysis, the protein was detected to have 12 transmembrane regions (Figure 2).

The amino acid sequence predicted from *OsNHX1* was detected to have significant identity with the amino acid sequence of NHX1 and mammalian Na⁺/H⁺ antiporter (NHE)(Table 1). Specifically, high identity was seen in the transmembrane region supposed to be involved in ion transport (Figure 3).

These three proteins (NHX1 from budding yeast, NHE6 from mammals, and OsNHX1) turned out to form a cluster, according to the dendrogram formed for various Na⁺/H⁺ antiporters reported to date (Figure 4). The OsNHX1 protein of the present invention is expected to be expressed in intracellular organs, such as vacuoles, and play an important role in the Na⁺ transport of the vacuolar membrane, due to the report that NHX1 protein is expressed in the late endosome (Nass, R. and Rao, R. (1998) J.Biol.Chem. 273:21054-21060) and the indication that NHE6 protein is also expressed in the cell (Numata, M., Petrecca, K., Lake, N. and Orlowski J. (1998) J.Biol.Chem. 273:6951-6959).

Further, the present inventors succeeded in obtaining transgenic plants by transferring the isolated *OsNHX1* gene into the rice callus and redifferentiating them utilizing Agrobacterium method.

The present invention relates to a novel Na⁺/H⁺ antiporter derived from monocotyledoneae and the DNA coding said antiporter, as well as methods for production and use, especially for the

production of salt tolerant plants using same.   More
specifically, the present invention provides the following:
   (1) a DNA selected from the group consisting of:
       (a) a DNA encoding the protein consisting of the amino acid
sequence described in SEQ ID NO: 2, and
       (b) a DNA comprising the coding region of the base sequence
described in SEQ ID NO: 1;
   (2) a DNA encoding the Na^+/H^+ antiporter derived from
monocotyledoneae selected from the group consisting of:
               (a) a DNA encoding the protein consisting of the
amino acid sequence described in SEQ ID NO:2, wherein one or
more amino acids are substituted, deleted, inserted and/or added,   ,
and
               (b) a DNA hybridizing under a stringent conditions to the
DNA consisting of the base sequence described in SEQ ID NO:1;
   (3) the DNA of (2), wherein the monocotyledoneae is a plant
belonging to the *Gramineae* family;
   (4) a vector comprising the DNA of (1) or (2);
   (5) a transformant cell having the DNA of (1) or (2), or the
vector of (4);
   (6) the transformant cell of (5), wherein the cell is a plant
cell;
   (7) a protein encoded by the DNA of (1) or (2);
   (8) a method for production of the protein of (7), which
comprises the steps of:
       cultivating the transformant cell of (5), and
   recovering the expressed protein from said cell or the
supernatant of the culture thereof;
   (9) a transformant plant comprising the transformant cell of
(6);
   (10) the transformant plant of (9), wherein the plant is a
monocotyledon;
   (11) the transformant plant of (10), wherein the plant is a

plant belonging to the *Gramineae* family;

(12) the transformant plant of (11), wherein the plant is rice;

(13) a transformant plant that is the offspring or clone of the transformant plant of any of (9) to (12);

(14) a material for the breeding of the transformant plant of any of (9) to (13);

(15) an antibody that binds to the protein of (7);

(16) a nucleic acid molecule that hybridizes with the DNA described in SEQ ID NO: 1, and which has a chain length of at least 15 nucleotides.

The present invention provides a novel Na^+/H^+ antiporter derived from monocotyledoneae, as well as a DNA encoding the same.   The base sequence of the cDNA encoding the Na^+/H^+ antiporter "*OsNHX1*", derived from rice and isolated by the present inventors, is indicated in SEQ ID NO: 1.   The amino acid sequence of the protein encoded by the cDNA is described in SEQ ID NO: 2.

The "*OsNHX1*" gene showed significant identity with many known amino acid sequences of the Na^+/H^+ antiporters, and especially high identity was observed at sites related to ion transport.   This finding suggests that "OsNHX1" protein plays an important role in Na^+ transport in rice.   It is supposed that Na^+/H^+ antiporters of plants are involved in the securement of osmotic pressure balance in the plant body under a high salinity stress.   Thus, it is anticipated that the "*OsNHX*" gene especially can be applied to production of salt tolerant cultivars.

Not only "OsNHX1" protein, but also proteins with equivalent functions, are included in this invention.   The term "proteins with equivalent functions to 'OsNHX1' protein" herein means that the object protein functions as an Na^+/H^+ antiporter.   The activity of an Na^+/H^+ antiporter can be detected, for example, by detecting the H^+ ejection from the biomembrane

vesicle due to addition of Na^+ as the recovery of fluorescence, by monitoring H^+ concentration gradient between isolated biomembrane vesicle formed by H^+-ATPase as the fluorescence extinction of acridine orange (Fukuda, A., Yazaki, Y., Ishikawa,

5   T. Koike, S., and Tanaka, Y. (1998) Plant Cell Physiol. 39:196-201).

     In one embodiment, the protein with equivalent function to "OsNHX1" is a mutant protein having amino acid sequence with one or more amino acid substitution, deletion, insertion and/or

10   addition to the amino acid sequence of "OsNHX1" protein (SEQ ID NO: 2), and which retains equivalent functions with "OsNHX1" protein. Such proteins can be prepared, for example, according to the following method. A method inducing mutations in the amino acid of "OsNHX1" can be mentioned as one method well known

15   to ordinary skilled in the art. That is, one ordinary skilled in the art can prepare a modified protein with equivalent functions to "OsNHX1" by modifying the amino acid sequence of "OsNHX1" protein (SEQ ID NO: 2). For example, by utilizing a site-directed mutagenesis method (Kramer, W. & Fritz, H. -J.

20   "Oligonucleotide-directed construction of mutagenesis via gapped duplex DNA" Methods in Enzymology 154:350-367, 1987) and such with the purpose to increase protein activity or the like. Mutations of amino acids also happen to occur in nature. The protein of this invention include proteins having amino acid

25   sequence with 1 or more amino acids substitution, deletion, insertion or addition to the natural amino acid sequence of "OsNHX1" protein (SEQ ID NO: 2), and that retain equivalent functions to natural proteins, regardless whether they are artificial or derived from nature. There is no limitation on

30   the part or the number of amino acid in the protein to be modified, so long as the modified protein retains equivalent functions with the natural "OsNHX1" protein. Generally, amino acidmodifications are done to amino acids within 100 amino acids,

preferably within 50 amino acids, much more preferably within 20 amino acids, and most preferably within 5 amino acids.

In another embodiment, the protein having equivalent functions with "OsNHX1" protein is a protein encoded by a DNA
5   derived from monocotyledoneae that hybridizes to the DNA encoding "OsNHX1" protein (SEQ ID NO: 1), having an equivalent function with "OsNHX1" protein.   Techniques such as hybridization techniques (Southern, E.M.: Journal of Molecular Biology, Vol.98, 503, 1975) and polymerase chain reaction (PCR)
10   techniques (Saiki, R.K. et al. Science, Vol.230, 1350-1354, 1985; Saiki, R.K. et al. Science, Vol.239, 487-491, 1988) can be mentioned as techniques known to those skilled in the art for preparing proteins.   That is, it is routine for a person skilled in the art to isolate a DNA with high identity to the
15   "OsNHX1" gene from rice or other monocotyledon and obtain proteins with an equivalent function to "OsNHX1" protein from that DNA, using the base sequence of "OsNHX1" gene (SEQ ID NO: 1) or parts thereof as a probe, and oligonucleotides hybridizing specifically to the base sequence of "OsNHX1" gene (SEQ ID NO:
20   1) as a primer.   Such proteins, derived from monocotyledoneae with an equivalent function to the "OsNHX1" protein, obtainable by hybridization technique or PCR technique, are included in the proteins of this invention.

Monocotyledoneae, preferably plants belonging to the
25   Gramineae family can be mentioned as plants used as the source of genes for isolation by hybridization techniques and PCR techniques.   For example, besides rice, barley (Hordeum vulgare), wheat (Triticum aestivum), maize (Zea mays) and so on can be mentioned, as plants belonging to the Gramineae family.
30   However, it is not limited to them.

Methods for isolating DNA encoding proteins with an equivalent function to the "OsNHX1" protein using the above-described techniques include, for example, but are not

limited to, the following. For example, hybridization of cDNA
or genomic libraries, prepared from monocotyledoneae with
probes (for example, DNA consisting of the base sequence
described in SEQ ID NO: 1 or parts thereof) labeled with ^{32}P

5   and such, is carried out. Conditions for hybridization using
^{32}P labeled probes are 25°C (without formamide) as a mild
condition and usually 42°C, employing hybridization solutions
(50% formamide, 5X SSPE, 2X Denhard's solution, 0.1% (w/v) SDS,
and 100 µg/ml of herring sperm DNA (Sambrook J, Fritsch EF,

10   Maniatis T (1989) Molecular cloning: A Laboratory Manual (Cold
Spring Harbor Lab., Cold Spring Harbor, NY), 2nd Ed.)).
Prehybridization is carried out at a minimum for more than an
hour, and hybridization is performed for 24 hours. Washing of
the hybridized filter is carried out at 25°C (wash solution:

15   2X SSC, 0.1%SDS) for a mild condition (a condition with low
stringency), at 42°C (wash solution: 2X SSC, 0.1%SDS) for an
ordinary condition, and at 56°C (wash solution: 0.1X SSC,
0.1%SDS) for a stringent condition (a condition with high
stringency).

20   If the protein encoded by the DNA isolated as above has
an equivalent function as "OsNHX1" protein, it generally shows
a high amino acid sequence identity with "OsNHX1" protein. The
term "high identity" as used herein refers to an identity higher
than at least 60%, preferably higher than 80%, more preferably

25   higher than 85%, and much more preferably higher than 90%. The
amino acid sequence identityis calculated, for example, by a
homology analysis program (Lipman, D.J. and Pearson, W.R. (1985)
Science 227, 1435-1441) supplied by GENETYX software (Software
development corporation).

30   The protein of the present invention can be prepared by
methods known to those skilled in the art as recombinant proteins
or as natural proteins. Recombinant proteins can be prepared,
for example, by inserting a DNA encoding the protein of the

present invention into an adequate expression vector, transfecting an appropriate cell with the vector and purifying the protein from the transformant cell, as described later on. Alternatively, natural proteins can be prepared, for example,

5   by exposing cell extracts, prepared from cells that express the protein of the present invention (for example, rice cells), to affinity columns to which antibodies, prepared by immunizing appropriate immune animals with prepared recombinant proteins or partial peptides thereof, are attached, and purifying the

10   proteins bound to the column.

Additionally, the present invention provides DNAs encoding the proteins of the present invention described above.   The DNAs of the present invention includes genomic DNAs, cDNAs, and chemosynthetic DNAs and so on, and can be any DNA without

15   limitation, so long as it encodes a protein of the present invention.   The base sequence of the "*OsNHX1*" cDNA, included in the present invention, is shown in SEQ ID NO: 1.

The genomic DNA, as well as the cDNA can be prepared according to conventional methods, known to those skilled in

20   the art.   Genomic DNA, for example, can be isolated using PCR, by designing appropriate primers from the base sequence information of the gene of the present invention, and then screening a genomic library using the obtained amplified DNA fragment as a probe.   Alternatively, for example, it is possible

25   to isolate the cDNA from a cDNA library according to the same manner.

The DNA of the present invention can be, for example, utilized in preparation of recombinant proteins, as well as in production of transformant plants with salt tolerance.   In

30   preparing recombinant proteins, generally, a DNA encoding the protein of the present invention is inserted into an appropriate expression vector, the expression vector is transferred into an appropriate cell, the transformed cell is cultivated and the

expressed protein is purified.

Recombinant proteins can be prepared, for example, by transferring vectors, having DNAs encoding the protein of the present invention inserted therein, into cells, such as bacterial cells, like *E.coli*, yeast cells, insect cells, mammalian cells, and so on, by known gene transfer methods, like the electroporation method, the calcium phosphate transfection method and such, then expressing the recombinant proteins in the cell. Recombinant proteins expressed in the host cell can be purified according to methods known to those skilled in the art. For example, it is possible to express the protein as a fusion protein, with glutathione S-transferase (GST), using vectors such as pGEX (Pharmacia) in *E.coli*, and purify it using a glutathione column (Shigeo Ohno and Yoshifumi Nishimura (1997) "Cell Engineering supplement: Protocol of protein experiments" Shujun-sha).

Moreover, to prepare transformant plants using the DNA of the present invention, a DNA encoding the protein of the present invention is inserted into an appropriate vector, the vector is transferred into a plant cell, and the obtained transformed plant cell is regenerated. The transfer of the plant expression vector into the plant cell can be done for example, according to the species, through methods utilizing Agrobacteriums or methods involving the direct transfer into the cell. Methods that utilize Agrobacteriums, for example, are methods of Nagel *et al.* (Microbiol.Lett. 67:325(1990)) and methods of Raineri et al. for rice (Bio/Technology 8:33-38(1990)). These are methods in which Agrobacteriums are transformed with plant expression vectors (pUC system and so on. For example, pCAMBIA vector (Medical Research Council), etc.), and the transformed Agrobacteriums are transferred to plant cells using standard methods, like the leefdisk method, the callus method and so on. Methods for the directly transferring a plant expression vector

into a cell include the electroporation method, the particle gun method, the calcium phosphate method, the polyethylene glycol method and so.

There is no limitation on the plant cells to which vectors of the invention may be transferred, but monocotyledonous, preferably plants belonging to the *Gramineae* family are mentioned. Plants, like maize except rice, can be mentioned as plants belonging to the *Gramineae* family. Incidentally, the "plant cell" of the present invention includes various forms of plant cells, such as suspension culture cells, protoplasts, a section from the leaf, callus, and so on.

For example, methods, like the callus differentiation method (Kyozuka, J. and Shimamoto, K. (1991) Plant Tissue Culture Manual. Kluwer Academic Publishers, pp B1:1-16; Toki, S. (1997) Plant Molecular Biology 15:16-21), the differentiation method utilizing protoplasts (Shimamoto, K. *et al.* (1989) Nature 338:274-276; Kyozuka, J. *et al.* (1987) Mol.Gen.Genet. 206:408-413), and such in response to the kind of plant used, can be utilized to regenerate transgenic plants from transgenic plant cells to which vectors are introduced.

Transgenic plants produced in this way show high Na^+/H^+ antiporter activity as compared to wild-type plants, and are supposed to acquire salt tolerance thereby. Moreover, once a transformed plant transfected with the DNA of the present invention is obtained, it is possible to gain descendants from that plant body by syngenesis or agamogenesis. Alternatively, plants can be mass-produced from breeding materials (for example, seeds, fruits, ears, tubers, tubercles, stubs, callus, protoplast, etc.) obtained from the plant, as well as descendants or clones thereof. Plant cells transferred with the DNA of the present invention, plant bodies including these cells, descendants and clones of the plant, as well as breeding materials obtained from the plant, its descendant and clones,

are included in the present invention.

Such high Na^+/H^+ antiporter activity as compared to wild-type plants can be achieved either by high expression of Na^+/H^+ antiporter (change in quantity) or by expression of Na^+/H^+

5   antiporter with higher activity (change in quality), or may be a result of both.

Further, the present invention provides antibodies binding to the proteins of the present invention described above.   Both polyclonal antibodies and monoclonal antibodies are included

10   in the present invention.   Preparation of the antibody can be conducted according to methods known to those skilled in the art, for example, using methods of Harlow *et al.* (Harlow, E. and Lane, D. (1988) Antibodies: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).   Polyclonal

15   antibodies can be obtained by injecting fusion proteins, synthesized in *E.coli* or synthesized peptides, into a rabbit as antigens, obtaining rabbit antiserum, and purifying antibodies therefrom by affinity chromatography.   Monoclonal antibodies can be obtained by injecting antigens to mouse or

20   rats, cloning and preparing hybridomas, and subjecting thus obtained antibody to affinity chromatography.

Furthermore, the present invention provides nucleic acid molecules that hybridize with the DNA encoding the protein of the present invention, and which have a chain length of at least

25   15 nucleotides.   Such nucleic acid molecules can be used, for example, as probes to detect or isolate the DNA encoding the protein of the present invention, as well as primers to enhance such DNA.   Such nucleic acid molecules preferably hybridize specifically to the DNA encoding the protein of the present

30   invention.   The term "hybridizes specifically" as used herein means that it hybridizes to the DNA encoding the protein of the present invention but it does not hybridize to DNAs encoding other proteins under a normal hybridization condition,

preferably under a stringent condition for hybridization.

In addition, such nucleic acid molecules can be used as antisense oligonucleotides, ribozymes, and so on, that suppress expression of the protein of the present invention.
5 Derivatives and modified forms of the antisense oligonucleotides can be used in the same manner as the antisense oligonucleotide itself. The antisense oligonucleotide does not have to be completely complementary to the nucleotides constituting the given region of the DNA or mRNA, and may include
10 1 or more nucleotide mismatches, provided it can suppress expression of the protein. An antisense oligonucleotide and a ribozyme that suppresses expression of a protein of the present invention can be a very useful tool for the function analysis of the protein of the present invention.

15

Brief Description of the Drawings

Figure 1 shows the base sequence and the predicted amino acid sequence of the rice Na^+/H^+ antiporter (*OsNHX1*) cDNA. The
20 amino acid sequence is expressed in one letter notation.

Figure 2 shows the hydrophobicity plot of the amino acids of the OsNHX1 protein. The abscissa indicates the amino acid residue, and the ordinate indicates the degree of hydrophobicity. Predicted transmembrane regions are shown as boxed numbers.

25 **Figure 3** shows the amino acid sequence comparison between OsNHX1 and other Na^+/H^+ antiporters. Transmembrane regions (M3 to M6) are indicated above the sequence. Regarding the symbols under amino acids, "*" represents that all amino acids are conserved; ":" and "." represent that amino acid are similar.
30 ":" indicates much more similarity than those indicated by ".". The box with A represents the binding site of the specific inhibitor, amiloride, and the boxes with B represent sites with high identities to the mammalian Na^+/H^+ antiporter.

Figure 4 shows the result of phylogeny analysis of Na$^+$/H$^+$ antiporter using ClustalX (Thompson, J.D. *et al.* (1994) <u>Nucleic Acids Research</u>, <u>22</u>:4673-4680)(Neighbor Joining (NJ) method).

Figure 5 shows photographs indicating effects of hygromycin and NaCl on budding yeast *NHX1* mutant strain (*Δnhx1*) expressing *OsNHX1* gene. WT was K601 (wild-type) containing pKT10(+*HIS3*), *nhx1* was R100 (*Δnhx1*) containing pKT10(+*HIS3*), and *nhx1* GAP:OsNHX1 was R100 containing pKT10(+*HIS3*)+*OsNHX1*. Five-fold serial dilutions (starting at no dilution) of the indicated strains were grown at 28°C for 3 days on YPD agar medium supplemented with or without hygromycin or for 8 days on APG agar medium (pH 4.0) supplemented with or without NaCl.

Figure 6 shows a photograph of electrophoresis indicating results of Western analyses for OsNHX1 protein in a microsomal fraction of budding yeast *NHX1* mutant strain (*Δnhx1*) and a membrane fraction of rice. TM, TP, and PM indicate total membrane, tonoplast, and plasma membrane, respectively. The microsomal fraction of budding yeast was prepared from R100 and R100 into which pKT10(+*HIS3*)+*OsNHX1* had been introduced. The membrane fraction of rice was prepared from roots and shoots of young rice plants hydroponically cultivated for seven days. The proteins prepared (10 μg) were electrophoresed on a 10% SDS gel and subjected to Western analyses with OsNHX1 protein-specific antibody and vacuolar H$^+$-pyrophosphatase-specific (V-PPase-specific) antibody.

Best Mode for Carrying out the Invention

Any patents, patent applications, and publications cited herein are incorporated by reference.

The present invention will be specifically explained with reference to the following examples. However, it should be noted that the present invention is not limited by these examples.

Example 1-Cloning of the rice Na$^+$/H$^+$ antiporter gene

       A sequence having identity to Na$^+$/H$^+$ antiporter (NHX1) obtained from budding yeast was analyzed from the database for higher plants of GeneBank. A cDNA clone from the cDNA library

5   of rice panicle was identified. The amino acid sequence predicted from the clone had 37% identity with NHX1. It was presumed that the obtained cDNA clone did not have the full-length base sequence. Therefore, using the cDNA clone as a probe and using the cDNA library constructed from mRNA prepared from the

10 root of rice (*Oryza sativa* L. cv Nipponbare) seedling as the template, selection of a cDNA clone having the full-length insertion was performed.

       Rice seeds were imbibed overnight, and placed on cotton mesh suspended over a nutrient solution (0.5 mM NH$_4$H$_2$PO$_4$, 1 mM

15 KNO$_3$, 0.5 mM MgSO$_4$, 12.5 µM Fe-EDTA, 1 mM CaCl$_2$, micronutrients). Cultivation was performed 7 days with a cultivation condition: day(brightness 40 µmol m^{-2} s^{-1}) for 14 hours at 30°C, night for 10 hours at 25°C, humidity at 75%.

       Poly(A$^+$)RNA from the root of rice seedling was prepared

20 and size fractionated by 5 to 25% sucrose density-gradient by centrifugation. Then, a cDNA library was constructed from the fractions containing relatively large poly(A$^+$)RNAs (Tanaka, Y. *et al.* (1989) Plant Physiol. 90:1403-1407). Double stranded cDNA was synthesized from size fractioned poly(A$^+$)RNA by the

25 method of Gubler and Hoffman (Gubler, U. and Hoffman, B.J. (1983) Gene 25:263-269), using oligo dT as the primer. The sample was then size fractioned by high performance liquid chromatography (Tosoh, Tokyo, model CCPD,) using Asahipack GS710 column (Asahi Chemical Industry Co. Ltd., Tokyo; 2.5 X 50 cm). cDNAs larger

30 than 2kb were inserted to the *Eco*RI site of λgt11.

       Plaque hybridization was conducted using constructed λ phages having cDNA libraries, and cDNA clones that show identity with the NHX1 as probes. Selecting a vector with the longest

cDNA insert from the plaques that showed signal, cloning was performed by inserting the cutout cDNA into a pBluescript(KS+)vector (Stratagene). Confirmation that the obtained cDNA clone is a full-length cDNA was made by the signal

5   size from the Northern hybridization using RNAs extracted from the rice plant body and the obtained clone as the probe. All base sequence of the cDNA, to which the whole isolated gene (referred to as *OsNHX1*) is inserted, was determined (Figure 1).

10 Example 2-Base sequence and amino acid sequence analysis of *OsNHX1* gene

The full-length sequence was 2330 base pairs, the 5' untranslated region was 296 base pairs, the translated region was 1608 base pairs and the 3' untranslated region was 426 base

15 pairs. The protein encoded by *OsNHX1* was predicted to be 535 amino acids long, and the molecular weight was calculated to be 59,070 daltons. 59% of the predicted amino acids sequence was hydrophobic, 22% was neutral amino acids, and 19% was hydrophilic amino acids. Thus, the protein seemed to be highly

20 hydrophobic. The result of hydrophobicity analysis, by the method of Kyte and Doolittle (Kyte, J. and Doolittle, R.F. (1982) J.Mol.Biol. 157:105-132), is shown in Figure 2. Twelve transmembrane regions were detected by the method of TMpred program (Hofmann, K. and Stoffel, W. (1993) Biol.Chem.

25 Hoppe-Seyler 374:166).

Significant identity was detected for the amino acid sequence predicted from *OsNHX1* with the amino acid sequence of NHX1 and mammalian Na$^+$/H$^+$ antiporter (NHE)(Table 1; NHX1 in the table represents that derived from yeast [*S.cerevisiae*], NHE6

30 from human, and NHE1 to 4 from rat. The values on the table were calculated by the homology-analyzing program (Lipman, D.J. and Pearson, W.R. (1985) Science 227:1435-1441) of GENETYX (ver.10) software (Software Development Company)). Especially

high identity was observed in the transmembrane regions, which were suspected to be involved in ion transport (Figure 3). [83]LFFIYLLPPI[92], a part of the amino acid sequence of OsNHX1(residues 83-92 of SEQ ID NO.2), is very well conserved

5   in NHX1 and NHE and is expected to be the binding site of amiloride, an inhibiter of the eucaryotic Na^+/H^+ antiporter (Counillon, L. *et al.* (1993) <u>Proc.Natl.Acad.Sci.USA</u> <u>90</u>:4508-4512)(Figure 3A). In addition, the 6th and 7th transmembrane regions are well preserved in eucaryotic Na^+/H^+ antiporter and, thus, is predicted

10   to play an important role in the transport of Na^+ and H^+ (Orlowski, J. and Grinstein, S. (1997) <u>J.Biol.Chem.</u> <u>272</u>:22373-22376). The 5th and 6th transmembrane regions in the amino acid sequence of OsNHX1 showed high identity to these regions (Figure 3B). The above results indicate that the protein encoded by *OsNHX1*

15   has the activity of Na^+/H^+ antiporter.

Table 1

	OsNHX1	NHX1	NHE6	NHE1	NHE2	NHE3	NHE4
OsNHX1	100	29.5	33.0	30.1	29.4	26.7	27.7
NHX1		100	36.1	28.6	29.1	29.3	32.0
NHE6			100	31.9	29.1	31.8	28.6
NHE1				100	48.9	37.1	45.5
NHE2					100	44.7	66.0
NHE3						100	44.6
NHE4							100

Amino acid sequence identity of OsNHX1 to other Na^+/H^+ antiporters (%)

20   Dendrogram of various Na^+/H^+ antiporters reported to date, namely mammalian NHE, budding yeast (*S.cerevisiae*) NHX1 and NHA1, Sod2 (which is expected to be expressed on the plasma membrane of fission yeast, *S.pombe*), yeast (*Zygosaccharomyces rouxii*)

ZSod3, *E.coli* NhaA and NhaB, as well as OsNHX1 (noted as "OsNHX1" in the figures) made according to NJ method, revealed that three of them, that is NHX1, NHE6 and OsNHX1, form a cluster (Figure 4). It has been reported that NHX1 protein is expressed in the
5   late endosome (Nass, R. and Rao, R. (1998) <u>J.Biol.Chem.</u> <u>273</u>:21054-21060), and it was indicated that NHE6 protein is also expressed in the cell (Numata, M., Petrecca, K., Lake, N. and Orlowski, J., <u>J.Biol.Chem.</u> <u>273</u>:6951-6959). Therefore, it is expected that OsNHX1 protein is expressed in the intracellular
10   organs, like the vacuole and so on, and plays an important role in Na^+ transport in these organs.

<u>Example 3-Production of transformed rice expressing rice Na^+/H^+</u>
<u>antiporter gene</u>
15   *OsNHX1* inserted in the *Bam*HI site of pBluescript KS+ (STRATAGENE) was excised with *Kpn*I and *Not*I. Then, OsNHX1 was inserted downstream of the cauliflower mosaic virus 35S promoter of pMSH1 (for high expression) and pMSH2 (for repressed expression), both of which are derived from Ti-plasmid and are
20   transferred with kanamycin resistance gene and hygromycine resistance gene (pMSH1: Kawasaki, T. *et al.* (1999) <u>Proceedings</u> <u>of the National Academy of Sciences of the U.S.A.</u> <u>96</u>:10922-10926; pMSH2: the multi cloning site has the opposite direction compared to pMSH1). Using the constructed vector, the rice callus was
25   transformed with *Agrobacterium tumefaciens*. The callus was induced from the seed, and screened after the infection with *Agrobacterium* was complete using hygromycine. The screened callus was differentiated to obtain the transformant plant. Transformation and differentiation were basically performed
30   according to the method of Toki (Toki, S. (1997) <u>Plant Molecular</u> <u>Biology</u> <u>15</u>,16-21).

Example 4-Functional complementation experiments in yeast

Experiments for functional complementation by *OsNHX1* gene were performed using budding yeast vacuolar Na$^+$/H$^+$ antiporter gene *NHX1* mutant strain (*Δnhx1*, R100) (Nass, R. et al., (1997) The Journal of Biological Chemistry 272, 26145-26152). Budding yeast was cultured in YPD medium, SD medium, or, in the case of NaCl treatment, APG medium. *OsNHX1* gene was inserted downstream of the GAP promoter of pKT10 vector, into which *HIS3* gene was inserted. The resulting vector was introduced into budding yeast by lithium method. NaCl- and hygromycin-sensitivity of *Δnhx1* was recovered by overexpressing *OsNHX1* gene (Figure 5). Thus, it was confirmed that *OsNHX1* gene encoded a protein having vacuolar Na$^+$/H$^+$ antiporter function.

Example 5-Localization of OsNHX1 protein

A peptide synthesized on the basis of the carboxyl-terminal 16 amino acids of the amino acid sequence deduced from *OsNHX1* gene was injected into a rabbit as an antigen. Antisera obtained were purified by affinity chromatography to prepare OsNHX1 protein-specific polyclonal antibody. Antibody preparation was requested from Sawady Technology Co., LTD. By Western analyses using the OsNHX1 protein-specific antibody obtained, it was confirmed that OsNHX1 protein was largely localized in the tonoplast fraction (Figure 6). Thus, it was confirmed that OsNHX1 protein was a vacuolar Na$^+$/H$^+$ antiporter.

Industrial Applicability. According to the present invention, it is expected that isolated Na$^+$/H$^+$ antiporter gene can render salt tolerance to the plant by expressing it in the plant. Therefore, it may conduce, for example, an increase in the harvest of crops, due to improvements in salt tolerance, by transfer into useful crops such as rice, which will make them resistant to harm by salt in dry land and such.